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13. ABSTRACT ***(Maximum 200 Words)***

We hypothesize that alterations in the regulation of growth by cAMP during mammary tumor progression are related to MAP kinase (ERK, JNK, p38) signaling modules known to be affected by cAMP and pertussis toxin (PT)-sensitive G proteins. cAMP stimulates normal cell mammary epithelial cell (MEC) proliferation and inhibits proliferation of hormone-independent mammary tumor cells suggesting it acts as a proliferation switch. Mammary epithelial cells from normal mouse mammary glands were compared to pregnancy-dependent (PDT) and ovarian-independent (OIT) mouse mammary tumors in serum-free, collagen gel cell culture. We find that the ERK cascade is only permissive for proliferation of normal, PDT, and OIT. cAMP stimulates or inhibits (OIT) proliferation via nonERK PT-sensitive pathways not involving insulin or protein kinase A activation of CREB phosphorylation. However, in OIT cAMP inhibited JNK activity implying involvement in cAMP growth inhibition. Examination of other MAPKs showed that neither direct activation or inhibition of the p38 MAP kinase correlated with stimulatory or inhibitory effects of cAMP on growth. However, inhibitors of p38 activity alone could stimulate growth and synergize with cAMP in normal MEC growth. In addition, OIT expressed an elevated level of active p38 whose inhibition caused growth suppression. Thus, p38 is associated with growth inhibition in normal MEC and growth stimulation in OIT. P38 is identified as an important mitogenic switch pathway related to cAMP mitogenesis. Examination of lysophosphatidic acid (LPA) signaling showed that it can stimulate MAPK activity and CREB and ATF2 phosphorylation, thus interacting with the cAMP pathway. H-89, a cAMP-dependent protein kinase A (PKA) inhibitor also stimulated proliferation of normal mammary epithelium but not OIT and could potentiate cAMP mitogenesis suggesting that nonPKA pathways are critical for cAMP mitogenesis. Thus, cAMP appears to stimulate or inhibit proliferation through pathways independent of PKA, ERK, and JNK. In contrast, p38 emerges as a potential critical mitogenic pathway involved in proliferation and antiproliferation.

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Walter Ingram 11/19/01

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Abbreviations

cAMP, 3'-5' cyclic adenosine monophosphate
CREB, cAMP response element binding protein
EGF, epidermal growth factor
ERK, extracellular regulated mitogen-activated protein kinase (MAP kinase)
ECL, enhanced chemiluminescence
IGF-I, insulin-like growth factor I
JNK, c-jun protein kinase
LPA, 1-oleoyl-lysophosphatidic acid
MAPK, Mitogen activated protein kinase
MEC, mammary epithelial cell
MEK, MAP kinase kinase
NMEC, normal mammary epithelial cell
OIT, ovarian-independent mammary tumor
PD, PD098059 (MEK inhibitor)
PDT, pregnancy-dependent mammary tumor
PKA, protein kinase A
PT, pertussis toxin
SPH, sphingosine-1-phosphate

Introduction

Changes in signal transduction networks occur during mammary tumor progression that affect the proliferative response of the cells to exogenous factors. These alterations may be related to the loss of hormonal regulation of breast cancer which has a major impact on prognosis and therapy of breast cancer. The goal of this research was to identify the mechanisms underlying the difference in the proliferative response to 3'-5' cyclic adenosine monophosphate (cAMP) that we observed in our rodent model systems (Imagawa *et al.* 1992). cAMP is a potent mitogen for normal mammary gland epithelium, weakly mitogenic for hormone-dependent mammary tumors and growth inhibitory to hormone-independent mammary tumors. cAMP-stimulated proliferation was inhibited by pertussis toxin (PT) in normal mammary epithelium but neither the inhibitory effect of cAMP nor basal cAMP-independent proliferation of hormone-independent mammary tumors was affected by PT (Imagawa *et al.* 1995). PT is a bacterial toxin that ADP-ribosylates Gαi subunits and blocks activation of receptor-coupled heterotrimeric Gαiβγ proteins. These findings indicate that through postreceptor crosstalk, pertussis toxin-sensitive Gαiβγ pathways modulate cAMP-mediated proliferation.

These preliminary results led to the hypothesis that a critical alteration in growth regulation related to signaling pathways affected by cAMP and pertussis toxin-sensitive G proteins occurs during transformation and progression of mammary tumors from hormone-dependent to hormone-independent growth. Furthermore, these effector pathways could play a pivotal role in coordinating multiple growth-stimulatory pathways and can modulate the hormonal responsiveness of mammary epithelium. An in vitro approach has been followed, taking advantage of our serum-free, primary cell culture system, to examine intracellular kinase pathways that may be altered during progression from normal mammary epithelium to hormone-independent mammary tumors.

Body

Materials and Methods

Reagents. Cell culture: Ham's F-12, Medium 199, and Dulbecco's Modified Eagle's medium (DMEM) were from GIBCO/BRL (Grand Island, NY); collagenase (CLS Type 2) was from Worthington Biochemical Co. (Freehold, NJ), Percoll was from Pharmacia Biotech (Piscataway, NJ). Rat tail collagen, solubilized in acetic acid, was prepared as described previously (Richards *et al.* 1983). Antibodies: nonspecific (i.e. total) antibodies to ERK 1 (C-16), ERK 2 (C14) and total and phosphospecific antibodies to jun kinase (JNK), CREB, and ATF-2 were from Santa Cruz Biotechnology (Santa Cruz, CA); total and phospho-specific antibodies to p38 and AKT and phosphospecific antibodies to ERKS 1, 2 were from New England Biolabs. Biochemicals: EGF was from Collaborative Research (Waltham, MA) and Protein A and G agarose was from Sigma Chemical Co (St. Louis, MO). Inhibitors to MEK1 (PD 098059), p38 kinase (PD16936, SB202190), protein kinase A (H-89) were from Calbiochem (San Diego, CA). Protein A and G agarose, dibutryl cyclic AMP (cAMP), pertussis toxin, 1-oleoyl lysophosphatidic acid (LPA) were from Sigma Chemical

Co. (St. Louis, MO) or Avanti Biochemicals (Birmingham, AL). LPA was prepared by brief sonication of an aqueous suspension in Saline A containing fatty acid-free bovine serum albumin (0.1 mg/ml).

MAP kinase substrate peptide containing the MAP kinase consensus phosphorylation sequence (amino acids 95-98 of MBP) was from Santa Cruz Biotechnology. [(g32P]-ATP was from DuPont-NEN. Gst-c-jun fusion protein substrate was prepared using a fusion construct provided by Roger Davis.

Cell culture and tissues. Ovarian-dependent mammary tumors (PDT) were transplanted into cleared fat pads and mice supplemented with estradiol and progesterone to induce tumor growth. Ovarian-independent mammary tumors (OIT) were raised by subcutaneous transplantation of tumor pieces in virgin DDD mice. These hormone-independent tumors grow rapidly in virgin hosts. Normal tissues were from mature virgin Balb/cAnNCrlBR mice obtained from Charles River.

Normal and tumor tissues were dissociated with collagenase (0.1%) and purified epithelial cells obtained by Percoll gradient centrifugation as described previously (Richards *et al.* 1983). For growth experiments, cell organoids were cultured for 10 days within collagen gels as described (Richards *et al.* 1983). The basal medium used for cell growth was composed of a 1:1 (v:v) mixture of Ham's F-12 and DMEM buffered with 20 mM HEPES and 0.67 g/l sodium bicarbonate, and supplemented with 10 μg/ml insulin, 100 U/ml soybean trypsin inhibitor, 1 μg/ml ∂-tocopherol succinate and other additives as indicated. Cell number was determined by fluorometric DNA assay using diaminobenzoic acid (Hinegardner 1971) and standard curves using diploid tumor cells.

For kinase assays, cells were cultured on collagen I-coated 6 cm petri dishes at 3-5 million cells per plate. For some experiments, cells were cultured in collagen gels. Cells were cultured in culture medium containing porcine serum (5%), EGF (10 ng/ml), and insulin (10 μg.ml) for 3-4 days until at least 75% confluency was achieved. The cultures were then washed and cultured in serum-free medium (above) for 2 days prior to the addition of test factors. cAMP (100 μg/ml) was added in combination with the cAMP phosphodiesterase inhibitor RO-20-1724 (10^{-5} M, Calbiochem). Pertussis toxin (100 ng/ml) was added overnight (to allow activation by the cells) prior to the addition of test factors. Kinase inhibitors SB202190 (p38), PD98059 (MEK/ERK), H-89 (PKA) were added 2 hrs prior to cAMP or lysophosphatidic acid (LPA) except where indicated. Pertussis toxin (Gαi) was added overnight.

Preparation of Cell Extracts. After incubation for various times, collagen gel cultures were terminated by aspiration of the culture medium followed by blotting of the gels on filter paper and transfer of the dehydrated gels to 0.6 ml of lysis buffer containing 20 mM Tris (pH 7.4), 0.3 M NaCl, 50 mM NaF, 2 mM EDTA, 1% (v/v) Triton X-100, 25 mM b-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10% glycerol, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, 2 μM benzamidine, 5 μg/ml aprotinin. The lysates were mixed by vortexing and left on ice for 60 min. before centrifugation (15 min, 13,000 x g, 4° C). Cells cultured on collagen-coated plates were terminated by quick freezing on dry ice followed by direct lysis on ice with 0.35 ml of lysis buffer. The cells were scraped and transfered,

1.5 ml microcentrifuge tubes and incubated on ice for 30 minutes. Extracts were obtained by centrifugation at 13,000 x g for 15 minutes. Supernatants were used for immunoprecipitations and western blot analysis. Protein concentration was determined using the BCA assay by Bradford.

Western Immunoblotting. Sample lysates containing 20-40 μg of total protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. Membranes were blocked with TBST buffer containing 5% (w/v) dry milk (or 1% BSA for phosphotyrosine antiserum) and 0.1% Tween and incubated with the manufacturer's recommended dilution of antibody (dilutions were adjusted as necessary). For all immunoblotting, a horseradish peroxidase-conjugated secondary antibody was utilized to allow detection of the appropriate bands using enhanced chemiluminescence (ECL, Amersham Corp.). Membranes were stripped for reprobing with another antisera by incubating at 65° C for 30 min. in Tris (63 mM, pH 6.7), 2% SDS, and 100 mM 2-mercaptoethanol then washed in TBST and reblocked. For data analysis, ECL-detected bands were scanned using a Molecular Dynamics Personal Densitometer using ImageQuant software.

ERK Kinase Assay. MAPK assays were performed using an immune complex kinase filter assay as described previously with minor modifications (Reuter *et al.* 1995). Briefly, lysate protein (0.3 mg) was immunoprecipitated (>90% efficiency) with Protein A-sepharose conjugated with 3 mg anti-MAP kinase rabbit polyclonal antibodies (ERK1 and ERK2). The Protein A was then resuspended in kinase reaction buffer containing 20 mM ATP, 0.25 mg/ml myelin basic protein peptide, and 10 mCi of [g32P]-ATP. After incubation, samples were centrifuged and aliquots spotted on p81 cation-exchange filter papers (Whatman)which were washed in with phosphoric acid and radioactivity was quantitated by liquid scintillation counting

JUN Kinase Assay. Jun kinase activity was determined by kinase assay of c-jun immunoprecipitates as described by Dhanwada et al.(Dhanwada *et al.* 1995). Briefly, c-jun was immunoprecipitated from cell lysates (0.15 mg protein) with JNK antisera conjugated to Protein G beads. Beads were then washed and incubated for 15 min in kinase buffer containing 10 mCi of [g32P]-ATP, and 8 mg of c-jun fusion protein substrate. The reaction was stopped by the addition of 2X sample buffer and the samples electrophoresed on 10% SDS PAGE gels. The gels were then stained with commassie blue, dried and exposed to x-ray film. Densitometry was performed on the substrate fusion protein band as described for ERKs above.

Results and Discussion

Objectives: (Figures, Page 20-24)

1. Role of the ERK mitogen activated protein kinase pathway in hormone, cAMP, and EGF mitogenesis.

Question: Is the ERK pathway required for mitogenesis by cAMP, P+PRL, or EGF?

Strategy: Specifically inhibit the pathway and determine the effect of this inhibition on the stimulation of proliferation by these factors:
Results: The participation of the ERK pathways in proliferation stimulated by hormones, EGF, and cAMP was determined in primary, serum-free culture experiments using normal and tumor tissues varying in their mitogenic response to these factors. PD098059 (PD) is a highly specific inhibitor of the ERK cascade (Dudley *et al.* 1995). It inhibits raf activation of MEK, the upstream kinase that phosphorylates and activates ERKs 1, 2 (referred to as ERKs). This inhibitor has been used to evaluate the role of this kinase cascade in the proliferative response to mitogens. PD inhibited proliferation stimulated by P + PRL, cAMP, and EGF in normal mammary epithelial cells (NMEC) (**Fig. 1**). In PDT cells, which are very mitogenically responsive to PRL and EGF, PD inhibited growth stimulated by these factors while the low or negligible growth response to cAMP alone in PDT made it difficult to evaluate its effect on cAMP mitogenesis in these cells. In OIT which grows in basal medium, PD inhibited growth completely at the highest concentration tested (Figs 1, 2, 3 of 1998 Annual Report)..
Conclusion: The ERK MAP kinase pathway is required for <u>full</u> mitogenesis by hormones and cAMP and for the growth of OIT.

Question: Does pertussis toxin (PT) inhibit growth stimulatd by cAMP and hormones?
Strategy: Add PT to cell cultures and determine the effect on growth stimulation by the above.
Results: We showed previously that pertussis toxin (PT) inhibited cAMP, and P+PRL-stimulated proliferation in normal mammary epithelium and found that it also inhibited PRL-stimulated growth in PDT. Although cAMP only slightly stimulated the growth of PDT, inhibition of growth in the presence of cAMP was observed. In contrast, the inhibitory effect of cAMP on the growth of OITs was not reversed by PT indicating that this inhibitory effect was not mediated by a PT-sensitive pathways (Figs 4, 5 of 1998 Annual Report).
Conclusion: PT inhibits proliferation stimulated by cAMP and hormones in NMEC and this pathway(s) appears to be preserved in PDT but lost in OIT.

Question: Do cAMP, hormones, and EGF stimulate ERK MAPK activity? This is expected if proliferation stimulated by these factors is sensitive to the MEK inhibitor, PD98059.
Strategy: Add these factors to cultured cells and monitor ERK activity
Results: Hormonal stimulation (PRL or P+PRL) of ERK activity was only weakly observed for NMEC and the time of peak activation varied in time course experiments (**Fig 2**) PRL did not stimulate ERK activity in PDT. Thus, the strong mitogenic response to PRL in PDT is not dependent on the activation of the ERK cascade.

cAMP had no effect on ERK activity in either PDT or OIT (Fig 12, 14 of 1998 Annual Report) but contrary to expectation, inhibited ERK activity in NMEC in spite of its mitogenic effect (**Fig 3**). In OIT where cAMP is growth inhibitory, cAMP had no effect on ERK activation indicating that its inhibitory effect on proliferation is not mediated by the ERK pathway. EGF, in contrast to hormones and cAMP, strongly activated ERK activity (Xing & Imagawa 1999).

PT inhibited basal ERK activity in NMEC but not PDT (Fig 7, 12 of 1998 Annual Report) or OIT (n=4 (not shown)). PT also inhibited PRL activation of ERK in NMEC (**Fig 2**).

Conclusions: Results from inhibitor and kinase activation studies indicate that the ERK pathway is only permissively required by cAMP and plays a minor role in hormone-stimulated growth. However, the ERK pathway is a major pathway for EGF mitogenesis. For cAMP in particular, nonERK signaling pathways mediate the mitogenic or antimitogenic (in OIT) response. ERKs are also not a PT-sensitive cAMP mitogenic pathway although PT inhibition of basal ERK activity may partially explain the inhibitory effect of PT on cAMP mitogenesis in NMEC.

Question: Is the insulin/IGF-I pathway the PT-sentitive pathway since insulin is a component of the basal culture medium?

Strategy: Culture cells in the presence and absence of insulin and determine the effect of cAMP and PT on growth and ERK activation.

Results: Proliferation. Another pathway that might be involved is the insulin/IGF-I pathway since PT inhibited cAMP stimulation of proliferation in serum-free medium containing only superphysiological insulin (10 mg/ml). Insulin at this concentration is a permissive requirement for optimal growth of mouse mammary epithelium in response to all known growth-stimulating factors. We asked if the inhibitory effect of PT was due to the inhibition of insulin-like growth factor I receptor (IGF-IR) mediated signaling since insulin at the concentration used can bind to the IGF-IR. The effect cAMP (100 μg/ml) and PT (10 ng/ml) on growth and kinase activation was compared in NMEC cultured in collagen gels for 12 days in serum-free medium in the absence (I0) or presence (I10) of insulin (10 μg/ml) (Fig. 1, 1999 Annual Report). We showed that cAMP requires superphysiological insulin to optimally stimulate proliferation but PT sensitivity is insulin-independent. That PT can inhibit the proliferative effect (albeit much reduced) of cAMP in the absence of insulin implies that the PT-sensitive step is not related to insulin receptor or IGF-IR activation.

ERK activity. When ERK activity was examined by western immunoblotting of cell extracts with phospho-specific ERK antiserum, *basal* activity was similar (slightly higher in I10) and inhibited by PT in both I0 and I10 media (**Fig 4**. (Fig 2 of 1998 Annual Report)). This result implies that basal ERK activity does not require the insulin or IGF-I pathways. cAMP inhibited ERK activity as previously observed in I10 as well as I0 medium (**Fig 4**). PT inhibited *basal* ERK activity in both media but when cAMP was added, the inhibitory effect of PT was affected by the presence of insulin; PT inhibition of ERK activity was almost complete in I10 medium but relatively slight in I0 medium. Total ERKs were the same level in all lanes as revealed by immunoblotting with ERK antisera. Thus, PT inhibited ERK activity to a greater extent in I10 medium containing cAMP suggesting that cAMP sensitizes the ERK pathway to PT via an interaction with an insulin/IGF-I pathway.

Conclusions: PT inhibition of ERK activity and proliferation is not dependent upon the co-activation of an insulin/IGF-I pathway. But, in the presence of cAMP, a PT-sensitive insulin/IGF-I pathway may be involved that can lead to almost total inhibition of growth and ERK activation.

Question: Are the inhibitory effects of PD98059 or PT on cAMP mitogenesis independent of cAMP induction of PKA activity?

Strategy: Culture cells and examine the effect of cAMP and inhibitors on the phosphorylation of PKA substrates, CREB and potentially ATF-1 or ATF-2.

Results: We showed that cAMP stimulates the proliferation of NMEC without stimulating ERK activity. However, cAMP mitogenesis is inhibited by pertussis toxin (PT) and PD, a specific inhibitor of the MEK/ERK pathway. Both of these factors inhibited basal ERK activity suggesting that although cAMP did not activate MAP kinases, a threshold level of ERK activity was permissively required for cAMP mitogenesis.

We sought to determine if the effects of PT and PD could also be directed at the level of transcription factor activation, especially at transcription factors that are known to be activated by cAMP via phosphorylation by protein kinase A (PKA). cAMP response element binding protein (CREB) phosphorylation was examined in NMEC cultured in the presence of cAMP with or without inhibitors. We observed that cAMP stimulation of CREB phosphorylation was inhibited by the PKA inhibitor (H-89) as expected but not by PT or PD98050 (**Fig 5** (Fig. 1, 1999 Annual Report)). Phosphorylated ATF-1 is also detected by this antiserum and ATF-1 phosphorylation paralleled CREB phosphorylation. In contrast another transcription factor that potentially can be a substrate for PKA, ATF-2, was not phosphorylated after cAMP treatment (not shown). Interestingly, cAMP also stimulates CREB phosphorylation in OIT in which cAMP inhibits proliferation (n=3). We have not yet been able to assess the effect of CREB phosphorylation on transcriptional activation although our working assumption is that phosphorylation is indicative of activation.

Conclusion: These data show that PT or PD98050 do not inhibit cAMP-induced growth by inhibiting PKA-dependent transcription factor phosphorylation through cross-talk between their respective signaling pathways. Thus, the PT- or PD-sensitive cAMP mitogenic pathways are do not include the PKA-dependent pathway leading to CREB phosphorylation.

Question: Are other PKA pathways important for mitogenesis?

Strategy: Inhibit PKA activity by using a relatively specific inhibitor of the enzyme and determine the effect on cAMP-stimulated proliferation.

Results: Since we observed that H-89 could inhibit cAMP induction of CREB phosphorylation we tested the effect of H-89 on cAMP mitogenesis. Unexpectedly, H-89 in the absence or presence of cAMP stimulated rather than inhibited, the proliferation of NMEC cultured on the surface of collagen I-coated culture plates at (**Fig. 6**). When cells were cultured within collagen gels H-89 again stimulated proliferation in basal medium about 2-fold but when it was combined with cAMP, about 40% inhibition of cAMP mitogenesis was observed in 2 experiments. This difference needs to be explored further but may be related to the better extent of growth observed in the monolayer cultures or three-dimensional versus two-dimensional growth. These findings may indicate that a low level of basal PKA activity has a growth inhibitory activity in cultures of NMEC (not related to CREB since CREB is not activated) while in the presence of cAMP it partially mediates cAMP mitogenesis although the variation in this response necessitates further studies. In OIT, inhibiting PKA with H-89 (seen as an inhibition of cAMP-induced CREB phosphorylation) does not result in growth stimulation or reverse cAMP inhibition of growth indicating that a nonPKA pathway is involved in cAMP inhibition of growth in OIT. H-89 has a Ki of 0.048 μM towards PKA which is at least 10-100X lower than the Ki for PKC, CK I and II, MLCK, and PKG suggesting that inhibition of these kinases is not involved in the stimulatory effect on growth. Inhibition of PKC would be expected to inhibit growth since activation of this kinase is associated with growth stimulation (Imagawa *et al.*

1990, Bandyopadhyay *et al.* 1995). These findings may strongly suggest that the effect of cAMP on proliferation is mediated by more than the PKA pathway and is the result of a balance between positive and negative influences .

Conclusion: We conclude that cAMP stimulates proliferation in NMEC or inhibits proliferation in OIT predominantly via PKA-independent pathways. PKA activation can have a negative effect on proliferation even in cells whose growth is stimulated by cAMP.

2. Role of c-jun kinase and p38 MAP kinase pathways in cAMP mitogenesis

Question: Does cAMP affect the activity of other MAP kinase pathways?

Strategy: Examine and compare the effect of cAMP on the c-jun kinase (JNK) and p38 MAP kinase pathways.

Results: The effect of cAMP on the activation of the stress associated protein kinases, p38 and JNK was also examined NMEC cultured I10 serum-free medium to compare the response of these kinases to the ERKs. Both activity assays and western immunoblotting of cell lysates with phospho-specific antisera against JNK and p38 (**Fig 7** (Fig 8, 10 1998 Annual Report) showed that cAMP ± PT had no significant effect on p38 activity while JNK activity was slightly inhibited by PT or cAMP (30% maximal inhibition in the presence of cAMP and PT) although examination of the variation in this inhibitory effect in other experiments indicates that this result is probably not significant. cAMP did not affect JNK activity in PDT (Fig 13, 1998 Annual Report) (p38 has not been examined) but appeared to inhibit JNK activation in OIT raising the possibility that this pathway may be involved in cAMP's inhibitory effect on proliferation (Fig 15, 1998 Annual Report). Therefore, the inhibitory effect of PT on cAMP-stimulated growth does not involve the JNK or p38 pathways.

Conclusion: cAMP does not activate p38 or JNK MAPK pathways in NMEC or the JNK pathway in PDT. Thus, these kinaseas are not directly involved in cAMP mitogenesis. However, cAMP inhibition of JNK activity in OIT raises the possibility that the JNK pathway plays a role in proliferation and inhibition of this pathway by cAMP leads to growth inhibition.

Question: Is the p38 MAPK pathway permissive for cAMP mitogenesis?

Strategy: Examine and compare the effect of inhibitors of p38 MAPK on MAPK activity and proliferation.

Results: Earlier we reported that the level of active or phosphorylated p38 in cells cultured within collagen gels, in basal serum-free medium is elevated in OIT compared to normal MEC and PDT (**Fig 8**). The total level of p38 (phosphorylated and nonphosphorylated) assessed by immunoblotting with p38 antisera showed that it was similar in normal and tumor cells. This finding suggested that p38 MAPK could be a key kinase in the growth regulation of aggressive hormone-independent mammary tumors.

The p38 inhibitors SB202190 (SB) and PD16936 were used to assess the effect of inhibition of the p38 pathway on cAMP and LPA mitogenesis. Similar to our experience with H-89, to our surprise this inhibitor stimulated the growth of normal MEC (**Fig. 9**) but inhibited the growth of OIT (**Fig. 10**). These two inhibitors markedly synergize with cAMP (and LPA discussed below) in stimulating the proliferation of normal MEC. PD16936 differs from SB in not significantly

stimulating proliferation alone but is a more potent synergist showing this effect at a concentration of 0.1 μM while SB requires higher concentrations, >1 μM. The stimulatory effect of these inhibitors on proliferation raises the possibility that the p38 pathway is inhibitory to growth and is related to the inherent growth-limited phenotype of normal cells. One caveat is that the inhibitors may affect the activity of kinases other than p38. A related inhibitor, SB203580, at concentrations greater than 1 μM has been reported to inhibit the AKT/PKB apoptotic pathway by inhibiting phosphoinositide-dependent protein kinase 1 which phosphorylates and activates PKB (Lali *et al.* 2000) or stimulate ERK activity.

Conclusion: The p38 MAPK pathway is negatively associated with proliferation of normal epithelium but positively associated with stimulation of the proliferation of OIT or advanced tumors. This pathway may be a proliferative switch capable of stimulating or inhibiting proliferation. cAMP pathways and the p38 MAPK pathway interact as shown in the inhibitor studies.

Question: Inhibitors of p38 synergize with cAMP in stimulating proliferation of NMEC and inhibit the proliferation of OIT. Do they affect the phosphorylation of MAPK and downstream transcription factors CREB and ATF-2 in NMEC and OIT/

Strategy: Add inhibitor SB202190 (SB) or PD16936 to cultured cells in dose-response and time course studies. Analyze MAPK or transcription factor phosphorylation by western immunoblotting

Results: SB alone is a potent inducer of p38 and pJNK phosphorylation in NMEC as assessed using phospho-specific antiserum in western immunoblots of cell lysates but a relatively weak inducer of ERK phosphorylation (**Fig. 11**). Time course experiments (5, 30, 120 minutes) showed that the effect of SB reached maximum at 5 minutes of treatment for ERK and 30 minutes for p38 and JNK. These effects may be non p38 specific effects of inhibitor since they were observed at a high concentration (25 μM). Another p38 inhibitor, PD16936 (0.1, 1, 5 μM), was tested alone and found to stimulate p38 and JNK phosphorylation at 1μM and 5 μM (not shown). ERK phosphorylation was observed only at the highest concentration (5 μM) tested, however, raising the possibility that this too is a nonspecific effect. Phosphorylation of p38 is not indicative of activation in the presence of inhibitor. We have attempted to assay p38 activity by immunoprecipitating an endogenous substrate of p38, MAPKAP2, but none of the available antisera were capable of efficiently immunoprecipitating the mouse protein.

SB alone stimulates dATF-2 phosphorylation but inhibited CREB phosphorylation in normal cells by 5 minutes of treatment (Annual Report 2000) (**Fig 12**). PD16936 (1-5 μM) also stimulated ATF-2 phosphorylation.

In OIT, SB stimulated p38 phosphorylation similarly to normal but did not affect ERK or JNK phosphorylation (Annual Report 2000). Thus the growth inhibitory effect of the inhibitor is most likely related to the p38 pathway, i.e. this is a growth-promoting pathway. Compared to NMEC, SB treatment did not affect CREB phosphorylation but stimulated ATF-2 phosphorylation (Annual Report 2000).

Conclusion: SB (or PD16936) stimulation of proliferation may involve the activation of JNK and ATF-2 in NMEC. These pathways are not activated by cAMP alone and could explain potentiation of cAMP-stimulated proliferation by these compounds. Increased phosphorylation of p38 by

inhibitor indicates that p38 may normally be under negative feedback control. The inhibition of CREB phosphorylation reinforces our previous conclusion that CREB is not significantly involved in cAMP mitogenesis. Since SB did not affect ERK or JNK phosphorylation in OIT, the inhibitory effect of SB on growth only correlates with the inhibition of p38 activity (although p38 phosphorylation was increased). Thus, in OIT and NMEC,.p38 is associated, respectively, with the stimulation or inhibition of proliferation.

3. Effect of lysophosphatidic acid on MAPK activity in normal MEC and OIT.

Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (SPH) are phospholipid mitogens that selectively affect normal and tumor MEC growth. LPA stimualtes NMEC and inhibits OIT growth in the same manner as cAMP (Imagawa *et al.* 1995). Its mitogenic effect was shown to be similarly PT sensitive ((Imagawa *et al.* 1995) and Annual Report 1999, Fig 7). We have attempted to gain further insight into growth- stimulatory and growth-inhibitory pathways by comparing the effect cAMP with that of LPA or SPH on kinase pathways. We have recently also examined the expression of the EDG family of receptors for phospholipids: SPH and LPA are the high affinity ligands for, respectively, EDG 1, 3, 5 and EDG 2, 4. Expression of EDG2 (lpa1) and SPH (lpb1, lpb3) were examined by ribonuclease protection assay in normal and tumor mouse mammary tissues. **Fig. 13** shows that these receptors are expressed in normal mammary tissue, in whole fat pads and in cleared fat pads showing that they are expressed in the adipose tissue compartment. Expression declines during mammary differentiation associated with pregnancy and lactation and during tumorigenesis being very weak to undetectable in OIT. Expression of other members of the EDG family have not yet been examined.

NMEC:

LPA (50 μM) and SPH (1-10 μM) were found to stimulate the phosphorylation or activity of ERK, JNK, and p38 MAPKs in NMEC (Annual Report 1999, Fig. 6. A-D). LPA treatment activated ERK and p38 relatively more than JNK. Examination of transcription factors showed that CREB phosphorylation was stimulated by LPA (Annual Report 2000, Fig 3) while ATF-2 phosphorylation was not significantly affected (n=2, not shown). PT, which inhibits LPA-stimulated proliferation of cells cultured within collagen gels also inhibited LPA activation of all of these MAPKs . The role of the ERK pathway was examined using the MEK inhibitor, PD9805. This inhibitor partially inhibited LPA stimulated mitogenesis (as initially shown in our Annual Report 1999, Fig 7) and ERK phosphorylation but stimulated basal p38 phosphorylation and did not inhibit LPA-induced p38 phosphorylation (**Fig 14**). LPA stimulation of CREB phosphorylation was also inhbited by PD98059 showing that CREB activation was downstream of a Gαi-coupled (PT sensitive) pathway leading to ERK activation (Annual Report 2000, Fig, 3).

LPA transactivation of the EGF receptor has been observed in other systems (Daub *et al.* 1997, Cunnick *et al.* 1998, Prenzel *et al.* 1999, Zwick *et al.* 1999). EGF can activate CREB phosphorylation in NMEC (Annual Report 2000, Fig, 3) raising the possibility that EGF receptor transactivation may be induced by LPA in these cells. Compound 56 (Calbiochem), an EGF receptor kinase inhibitor, inhibited of LPA-stimulated CREB phosphorylation (Annual Report 2000, Fig, 3) suggesting that EGF receptor transactivation may be occurring. We find, however, no evidence that

LPA treatment of cells induces EGF receptor phosphorylation as assayed by immunoprecipitation of the receptor and phosphotyrosine blotting (Annual Report 1999). This inhibitory effect of Compound 56 may be a nonspecific effect.

Inhibition of p38 MAPK activity by SB202190 or PD16936 did not inhibit LPA-induced proliferation, instead growth was stimulated in synergism with inhibitor as shown in **Fig. 9**. Repeat expeiments show that the synergistic effect on growth (Fig 9) can be significantly higher. However, inhibition of p38 activity by these inhibitors resulted in the stimulation of ATF-2 phosphorylation in the absence of any effect on CREB phosphorylation in basal controls. Thus, p38 is not required for LPA mitogenesis but may negatively mediate ATF-2 phosphorylation explaining why only CREB phosphorylation is significantly induced after LPA stimulation.

OIT:

We have begun studies examining the effect of LPA on OIT. To recapitulate, OIT growth is autonomous and independent of EGF in our culture system. EGF independence is explained by the low to undetectable level of EGF receptors in these tumors as assessed by western immunoblotting with EGF receptor antiserum under assay conditions where the receptor is easily detected in normal MEC (not shown). LPA inhibits OIT proliferation in vitro (Annual Report 1999, Fig. 8), but is not a significant activator or inhibitor of ERK, JNK, or p38 phosphorylation (not shown) although ERK and JNK phosphorylation have been observed in some experiments (Annual Report 1999, Figs. 9, 10). Thus, the inhibitory effect of LPA on proliferation does not correlate strongly with the inhibition of MAPK activity. This is similar to cAMP which has no effect (ERK, p38) or is inhibitory (JNK) to MAP kinases. However, a role for these MAPKs in the endogenous proliferation of OIT (growth in the absence of added mitogen) is suggested by the inhibitory effect of MAPK inhibitors (ERK and p38) on proliferation. We are drawn to the conclusion that the inhibitory effect of LPA and cAMP on proliferation is independent of direct stimulatory or inhibitory effects on MAP kinases (ERK, JNK) associated with proliferation control by mitogenic growth factors and cAMP in other systems

Research Accomplishments

1. Determined the effect of cAMP, LPA, EGF, and prolactin on the level and activation of ERK, JNK, and p38 MAPK in normal and tumor mammary epithelial cells differing in their mitogenic response to cAMP.
2. Evaluated the role of the ERK and p38 MAPK pathways in proliferation stimulated by cAMP and LPA by using inhibitors of these pathways.
3. Examined the activation of downstream transcription factors (CREB, ATF2) by cAMP, LPA, EGF.
4. Determined the effect of PT on cAMP-, and LPA, -stimulated proliferation, MAPK activity, and activation of transcription factors.
5. Examined the role of a PT-sensitive insulin receptor or IGF-I receptor activation in cAMP-induced mitogenesis.
6. Compared the level of raf-1 and B-raf expression in normal and tumor mammary epithelium.

7. Revealed novel and differential effects of pyridinyl-imidazole inhibitors on the proliferation of normal and tumor mammary epithelium and MAP kinase and transcription factor activation.

8. Revealed novel mitogenic effects of the protein kinase A inhibitor, H-89, on proliferation.

9. Extended studies on the lyosplipid, LPA, to show its effects on the phosphorylation of cAMP responsive transcription factors and interactions with pyridinyl-imidazole inhibitors.

Reportable Outcomes:

Manuscript (Appendix 2): Xing, C and Imagawa, W (1999) Altered MAP Kinase (ERK-1,-2) Regulation in Primary Cultures of Mammary Tumor Cells: Elevated Basal Activity and Sustained Response to EGF. Carcinogenesis 20, 1201-1208

Walter Imagawa, Charles Xing, Vadim Pedchenko, Cyclic AMP signaling during mammary tumorigenesis. Proceedings Era of Hope Meeting, US Army BCRP, Atlanta, GA June 8-11, 2000, Abst #M-29

Walter Imagawa, Vadim K. Pedchenko, Charles Xing. Lysophospholipid signaling in primary mammary epithelial cell proliferation. Abstract submitted to the Proceedings of American Association for Cancer Research, 2002.

Conclusions:

1. Although the ERK pathway is required for growth, a specific role for it in transducing the mitogenic response (stimulatory or inhibitory) to hormones, cAMP is not apparent. Thus, cAMP stimulation of proliferation of NMEC and inhibition of proliferation of OIT is not paralleled by effects on ERK activation. This interpretation does not exclude an important role for the ERK pathway since inhibition of this pathway does inhibit hormone and cAMP-stimulated proliferation and there can be crosstalk at points downstream of kinase activation. Rather they suggest that non ERK signaling pathways induced by ligand binding or protein kinase A activation are involved in the hormonal and cAMP initiation of the mitogenic response.

2. The inhibition of basal ERK activity by PT in insulin-containing medium bolsters our previous conclusion that basal ERK activity is permissive for cAMP mitogenesis and suggests that the PT-sensitive component of cAMP mitogenesis may be due to co-stimulation by a Gαi protein-coupled insulin or IGF-I receptor activation.

3. Examination of the JNK pathway showed that while EGF stimulates JNK activity in normal and PDT mammary epithelium, hormones and cAMP have no effect. This pathway appears not to be activated in response to these factors and probably is not directly invoked as part of their mitogenic response. For the OITs, however, cAMP appears to be inhibitory to JNK activation raising the possibility that this pathway may be involved in cAMP's inhibitory effect on proliferation.

4. Neither cAMP or PT regulate p38 activity in NMEC or OITs although basal activity appears to be elevated in OITs. Therefore, the inhibitory effect of PT on growth correlates best with an inhibitory effect on the ERK, not the JNK or p38 pathways in NMEC. The significance of elevated p38 activity in OITs is not known but inhibition of this pathway causes growth inhibition. In contrast, p38 inhibitors alone stimulate proliferation of NMEC suggesting that this pathway is normally inhibitory to proliferation.

5. Cyclic AMP stimulates proliferation by pathways not dependent upon the induction of CREB or ATF2 phosphorylation by PKA. However, activation of the ERK pathway by LPA or EGF leads to PT-dependent CREB phosphorylation. The p38 MAPK pathway is inhibitory to ATF2 phosphorylation and stimulatory to CREB phosphorylation.

6. The examination of raf expression patterns revealed that shifting ratios of raf-1 to B raf do not explain the different mitogenic effects of cAMP on normal MEC and mammary tumors. This suggest that the mechanisms responsible for the differential effect of cAMP on normal and tumor mammary epithelium lies either downstream of raf in signaling pathways or is not raf-dependent except in a permissive manner.

7. Mammary tissues contain receptors for bioactive phospholipids, LPA and SPH. The level of expression of these receptors is dependent upon physiological state indicative of regulation by hormones or cell differentiation. Receptors are present in stroma and epithelium suggesting a role in stromal/epithelial interactions.

7. The effect of LPA on signaling has been compared to cAMP. In spite of the similar effects these two factors have on the proliferation of normal and tumor MEC, we find that they have markedly different effects on kinase activation. LPA stimulates the activity of ERK, JNK, p38 while cAMP is inhibitory to ERK in normal MEC and JNK in OIT. The effect of LPA on kinase activity is PT-dependent for ERK but not JNK or p38. These results strengthen the interpretation that the positive and negative effect of cAMP on proliferation are not related by corresponding parallel effects on MAP kinases. However, a threshold level of ERK appears to be permissively required for cAMP mitogenesis.

8. Akt regulation is not a primary response related to proliferation control by cAMP or LPA.

9. These findings contribute to our knowledge of cAMP regulation of growth in normal and tumor MEC. Key findings are, a) cAMP stimulates proliferation via PT-sensitive non MAPK pathways which remain to be identified, b) p38 MAPK pathways may be an important in switching from proliferative to nonproliferative responses. Alterations in the activation in this pathway may contribute to tumor growth.

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Appendix 1, Figures

Figure 1

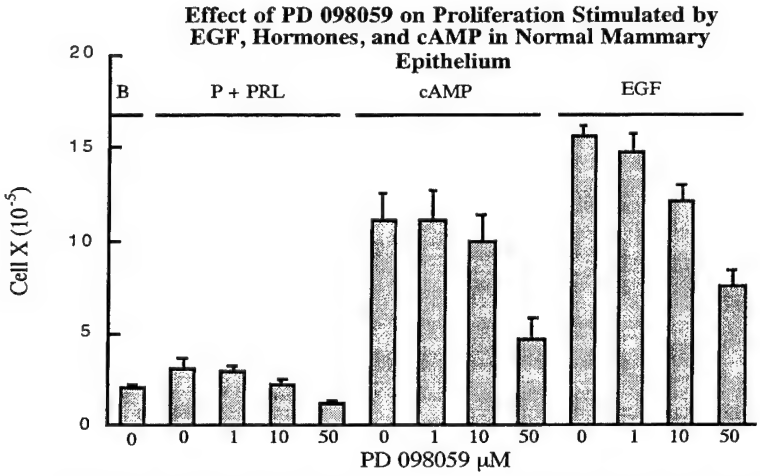


Fig 1. Cells were cultured in serum-free medium for 10 days before termination. Hormones (Progesterone (10^{-7}M) + Prl (1 ug/ml), EGF (10 ng/ml), or cAMP (100 ug/ml+IBMX (0.1mM)) were added in the absence and presence of PD. B is basal serum-free medium with no additives.

Figure 2

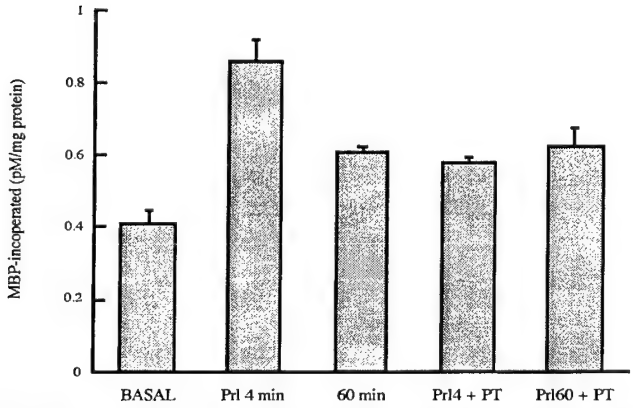


Fig.2 Effect of Prl and PT on ERK activity and in MEC from virgin mice. Cells were cultured within collagen gels in 2% serum then switched to serum-free medium for 24 hrs before PRL or PT addition for 4 or 60 min.

Figure 3

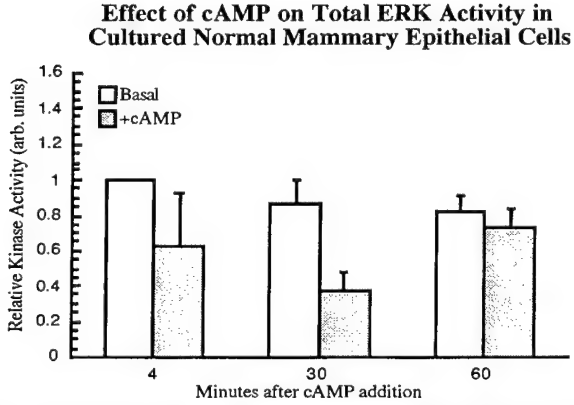
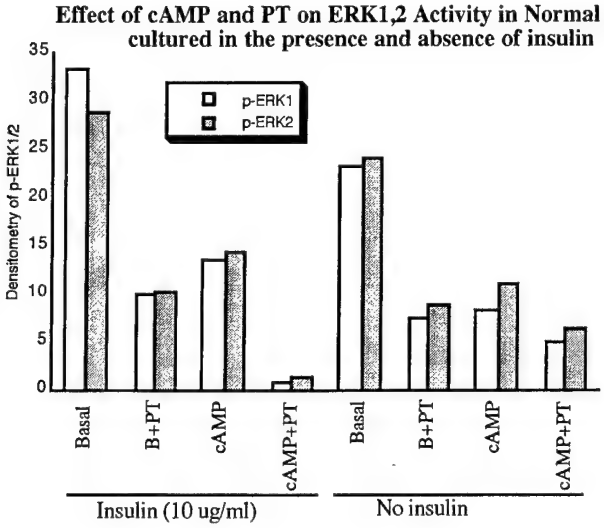


Fig. 3 Cultures were terminated at the indicated times after cAMP (100 ug/ml) addition. Average of 2 experiments. Data are normalized to Basal 4 min.

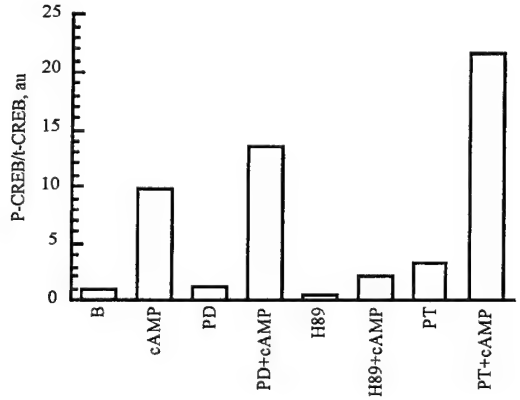
Appendix 1, Figures

Figure 4



Effect of insulin on cAMP and PT inhibition of ERK activity in cultured NMEC. Cells were cultured in collagen gels for 10 days (2% serum was added for the first 24 hrs prior to switching to serum-free medium with or without insulin (10 mg/ml). cAMP treatment was 30 min., and PT was added overnight. Activated ERK was determined by western immunoblotting with antiphospho-ERK antisera. Relative intensity of resultant bands detected by ECL was determined by scanning densitometry.

Figure 5



Effect of cAMP and inhibitors on CREB phosphorylation. Normal mammary epithelial cells (MEC) were cultured in collagen gels and after 3 days inhibitor addition was initiated. PT (50 ng/ml) was added overnight, and PD98059 (50 uM), H-89 (10 uM) were added 2 hrs prior to the addition of cAMP. After 30 min cAMP treatment, the gels were blotted on filter paper and transfered to lysis buffer on ice. Western immunoblot analysis of lysate proteins with phospho-specific CREB antiserum was followed, after stripping the membrane, by blotting with total CREB antiserum. Densitometry of bands was done and the results expressed as the ratio of phosphorylated CREB to total CREB (pCREB/tCREB) in arbitrary units. Representative of 3 experiments.

Appendix 1, Figures

Fig. 6

Effect of H-89 on proliferation. Normal mammary epithelial cells were cultured as monolayers in collagen-coated multiwells for 4 days in serum-free medium containing H-89 (10-0.1 μM) or cAMP in the absence and presence of H-89. Basal is no additive control, OT is starting cell number. Mean ± SD of triplicate cultures.

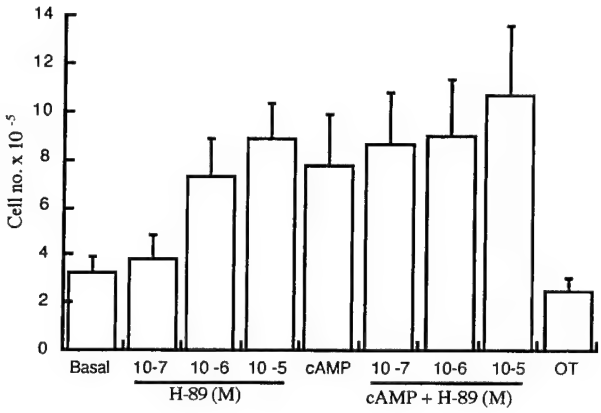


Fig. 7

NMEC were cultured in collagen gels (10 d) and treated with cAMP (30 min) ± PT. Western blot with phosphospecific antisera to JNK and p38 followed by ECLand densitometry.
Representative of 4 experiments.

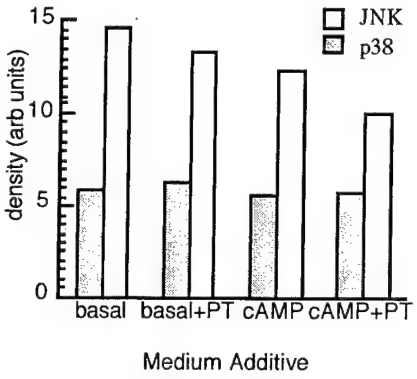
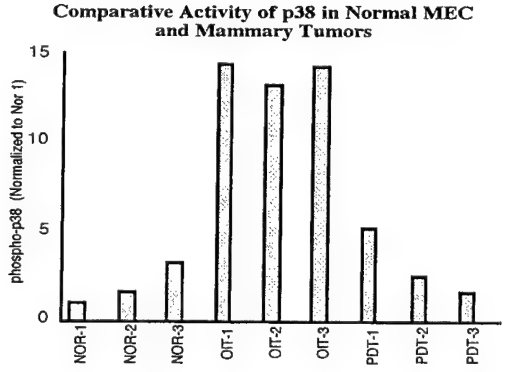


Fig. 8

Relative level of activated p38 in normal MEC and mammary tumors (OIT, PDT), Cells were cultured in collagen gels in serum-free medium for 10 days. Cell lysates were subjected to western immunoblotting with phospho-specific antiserum to p38. After ECL detection of bands, relative band density was determined by scanning densitometry and normalized to Nor-1.



Appendix 1, Figures

Fig. 9. Effect of inhibitors of p38 kinase on the proliferation of normal mammary epithelial cells. Cells were cultured in collagen gels in basal medium without or with the addition of cAMP, or LPA in the absence or presence of PD16936 (0.1-10 μM). Cells were cultured in basal medium containing SB202190 (0.5-25 μM). SB at 0.5 and 5 μM was also tested in combination with cAMP and LPA. Control basal cultures with no additions is the first column aon the left. Cells were cultured in triplicate for 10 days then terminated for DNA assay. Mean and SD of triplicates is plotted.

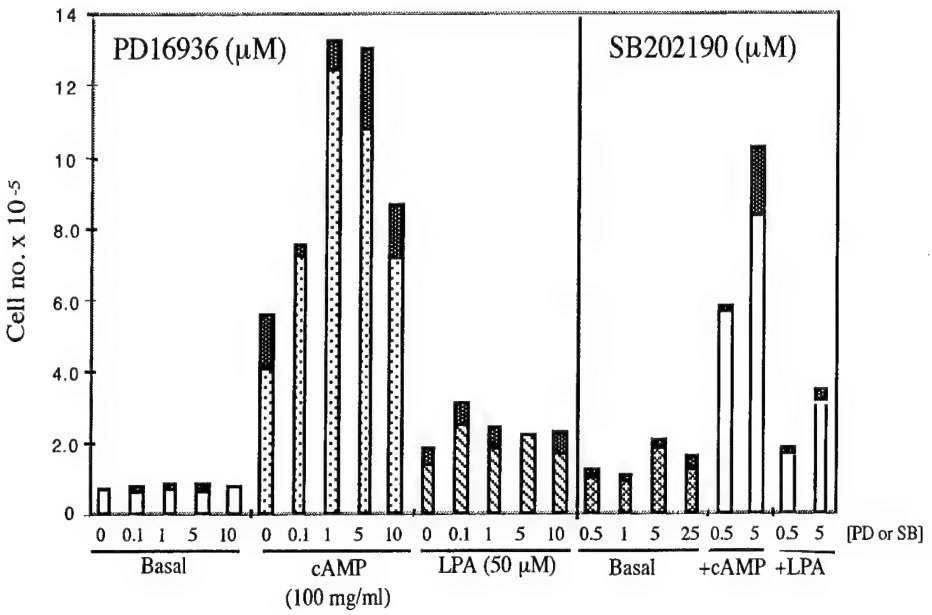


Fig. 10 Effect of the p38 kinase inhibitor SB202190 on the proliferation of OIT cells. Cells from ovarian-independent tumors were cultured within collagen gels in basal serum-free medium. SB202190 (5, 10, 25 μM) was added at the initiation of the cultures (OT is starting cell number) and the cultures were terminated for DNA assay after 10 days. The results of 2 separate experiments is plotted, mean and SD of triplicates.

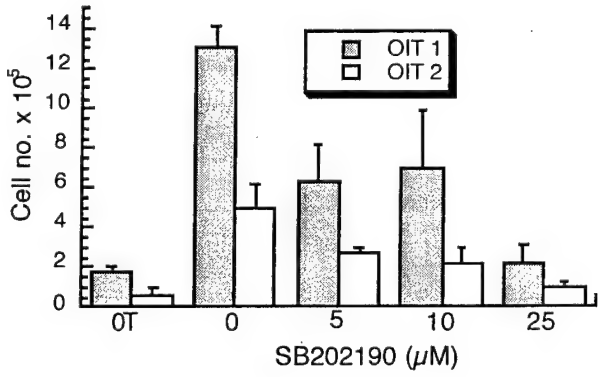
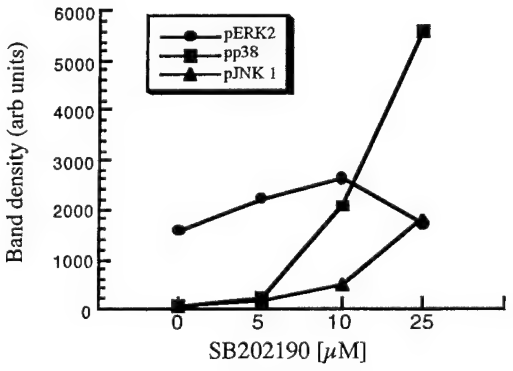


Fig. 11 Dose/response and time course effects of SB202190 on ERK2, p38, and JNK1 phosphorylation. Normal MEC were cultured in the absence or presence of SB202190 (5-25μM) for 120 minutes. Cell lysates were subjected to western immunoblotting with phospho-specific antisera. Plotted is band density after scanning densitometry. Representative of 3 experiments.



Appendix 1, Figures

Fig. 12 Effect of SB202190 on ATF2 and CREB phosphorlation. See Fig. 11 legend for details.

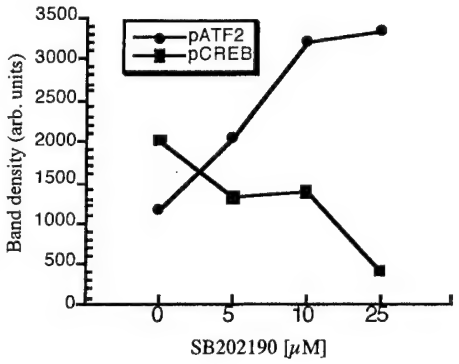


Fig. 13 Expression of phospholipid receptors in normal and tumor mouse mammary tissues. mRNA levels for LPA (lpa1) and sphinogsine-1-phosphate (lpb3, lpb1) receptors were assayed by RNP and normalized to actin. Mammary glands from virgin, midpregnant, lactating mice, cleared fat pads (CFP) and PDT and OIT tissues assayed (n=3, ave. Bands were scanned and normalized to actin. Expression in mammary glands form lactating (Lact) mice is artifically high due to decreased actin expression. lpa1 receptors were not detected in OIT.

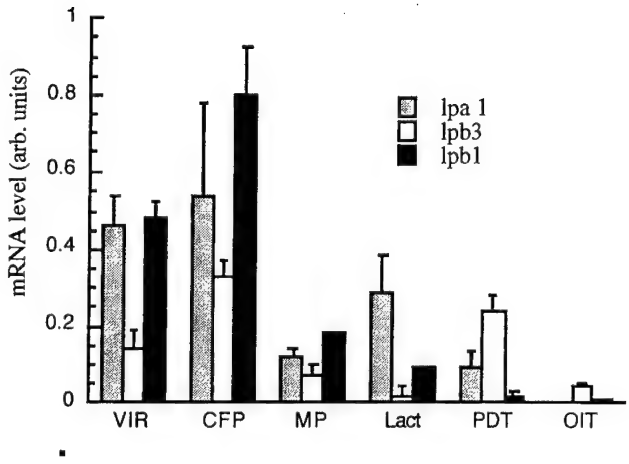
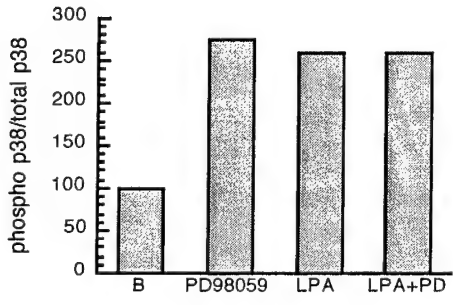


Fig. 14 Effect of PD98059 on basal and LPA-induced p38 phosphorylation. LPA (50 μM) was added alone and with PD98059 (50 μM, 2 hr preincubation) to cultured normal mammary epithelial cells. A representative western blot analysis. Bands were quantitated by scanning densitometry and p38 was normalized to total p38 for each treatment and all groups normalized to basal (B).



Carcinogenesis vol.20 no.7 pp.1201–1208, 1999

Altered MAP kinase (ERK1,2) regulation in primary cultures of mammary tumor cells: elevated basal activity and sustained response to EGF

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An elevation in total MAP kinase activity and expression has been observed in breast cancer tissue. However, the mechanisms underlying these changes in kinase activity and regulation by growth factors are not well characterized. In these studies, the effect of the potent mammary mitogen, epidermal growth factor (EGF), on the activation of the mitogen-activated protein kinases, ERK1 and ERK2 (extracellular regulated protein kinases 1 and 2, respectively), was compared in primary cultures of normal mouse mammary epithelial cells and in a hormone-responsive mouse mammary tumor. In normal epithelium, EGF stimulated an early rise in ERK activity at 4 min followed by a rapid decline, whereas a sustained (1 h) elevation of ERK activity was observed in the tumor cells. The time course of ERK activity in both cell types coincided with the phosphorylation state of the EGF receptor, suggesting that altered regulation of EGF receptor phosphorylation or EGF receptor turnover produces an enhanced ERK response to EGF in tumor cells. The MEK inhibitor, PD 098059 inhibited EGF-stimulated proliferation and ERK activity in a parallel, dose-dependent manner showing that ERK activation is at least permissive for the proliferative response to EGF. In addition, tumor cells showed a 4-fold elevation in basal (or ligand-independent) activity over normal cells without an increase in total enzyme level, and a preferential activation of ERK1 by EGF. These EGF-dependent and -independent changes in ERK regulation in the hormone-responsive mammary tumor underscore how multiple alterations in the regulation of this pathway may play a role in mammary tumorigenesis.

Introduction

Epidermal growth factor (EGF) is a potent mitogen for normal and tumor mammary epithelial cells. Overexpression of EGF and its receptor (EGFR or ErbB-1) has been observed in breast cancer, raising the possibility of potential autocrine growth regulation as observed in some breast cancer cell lines (1). Elevations in EGFR levels can also play a role in mammary tumor progression from hormone dependence to hormone independence, as shown in mouse mammary tumors (2) and in human breast cancer cells when the EGFR is overexpressed (3). In addition, an elevated level of EGFR is associated with a poor prognosis in breast cancer (1). The EGF receptor is a transmembrane tyrosine kinase that is activated by tyrosine autophosphorylation after ligand-induced dimerization (4). The ligand-bound receptor is capable of phosphorylating multiple signal transduction molecules, leading to the activation of multiple signal transduction pathways including protein kinase cascades (5). One of these kinase cascades is the *ras–raf* pathway leading to the activation of the extracellular regulated protein kinases (or ERKs), one of the mitogen-activated protein (MAP) kinase cascades. This pathway has been the subject of intense interest because of its role in the regulation of proliferation, differentiation and cell–matrix interactions. ERK1 and ERK2 are dually phosphorylated on threonine and tyrosine by the upstream MAP kinase, MEK. ERKs then phosphorylate and activate a variety of substrates including transcription factors, protein kinases and phosphotyrosine protein phosphatases leading to positive or negative regulation of signaling cascades (5).

Disruption of the regulation of the ERK pathway can predispose cells to undergo tumorigenic conversion as illustrated by the *ras* oncogene (6) which lies upstream of ERK, and transfection studies showing that constitutively active mutants of MEK can lead to *in vitro* transformation, increased sensitivity to, or independence from growth factors and tumor formation *in vivo* (7,8). Recent studies have shown an increase in the level and kinase activity of ERKs in human renal cell carcinoma (9) and breast cancer (10), indicating that deregulation resulting in overstimulation of this pathway may play a role in tumorigenesis.

These latter studies in primary human tumors did not address the regulation of the activation of the ERK cascade by exogenous factors. It was not possible to discern if an apparent constitutive elevation in basal activity was due to an inherent alteration in the regulation of the pathway or if the pathway was more sensitive to stimulation by an exogenous ligand. In our studies, we have sought to directly compare the effect of EGF on the activation of the ERKs in primary cell cultures of normal and tumor mammary epithelium to better recognize alterations that may occur during tumorigenesis. Primary culture using a biomatrix-based culture system was chosen to more closely approximate the *in vivo* state and as an alternative to immortalized cell lines adapted for growth on a plastic substrate. These experiments show that the mitogenic effect of EGF in normal and tumor mammary epithelium is dependent, at least in part, on ERK activation. Furthermore, mammary tumor epithelium may exhibit an elevation in basal ERK activity and sustained ERK activation by EGF, the latter sustained activation reflecting a difference in the regulation of EGF receptor activity. This altered regulation of the MAP kinase pathway may be an example of an alteration in regulation by growth factors that may occur in some breast cancers impacting both tumor growth and progression.

Materials and methods

Reagents

Cell culture: Ham's F-12, Medium 199 and Dulbecco's Modified Eagle's medium (DMEM) were from Gibco BRL (Grand Island, NY); collagenase

Abbreviations: EGF, epidermal growth factor; ERK, extracellular regulated protein kinase; MAP kinase, mitogen-activated protein kinase.

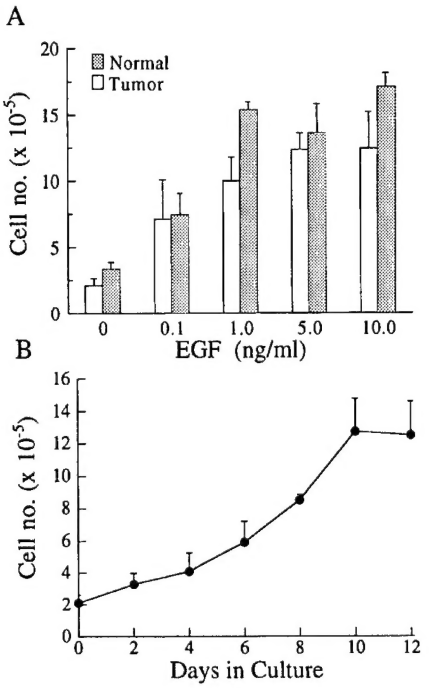

Fig. 1. The effect of EGF on the growth of mammary tumor cells. (**A**) Tumor cells were cultured for 10 days in serum-free medium containing different concentrations of EGF. (**B**) Time course of growth in the presence of EGF (10 ng/ml). Data are expressed as means ± SD of triplicates. Initial cell number was 2.5×10^5. Representative of three experiments.

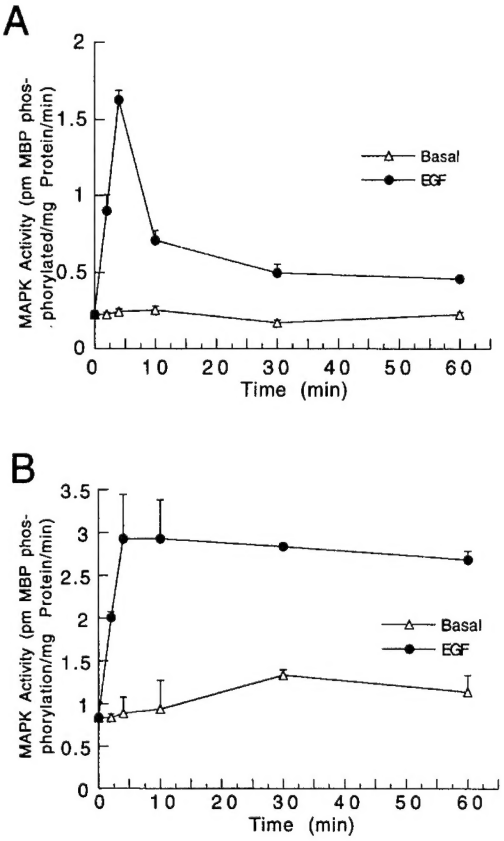

Fig. 2. Time course of MAP kinase activation in response to EGF (10 ng/ml) in (**A**) normal and (**B**) tumor mammary epithelial cells. Cells were plated on collagen-coated culture dishes and grown to near confluence in basal medium containing insulin plus BSA V (2.5 mg/ml) or 2% porcine serum for normal and tumor cells, respectively. Cultures were transferred to insulin-only medium for 24–36 h then treated with or without EGF for the desired time before lysis in extraction buffer. MAP kinase activity was assayed in duplicate in immunoprecipitates of cell extracts as described in Materials and methods. Averages and range of duplicates are plotted. Results are representative of three experiments for normal and tumor cells.

ERK2 activities determined after immunoprecipitation with combined ERK1 and ERK2 antisera. Figure 2A shows that in normal cells, ERK activity in immunoprecipitates rose to a peak at 4 min and declined rapidly within 10 min to a level ~2-fold higher than the basal activity. In comparison, tumor cells (Figure 2B) showed a 4-fold higher basal activity and a sustained increase in kinase activity in response to EGF. Although the fold activation by EGF was less than for normal cells (due to the elevated basal activity), the final level of activity was ~2-fold higher. At times >60 min, there was a gradual decline in ERK activation in tumor cells. The difference in the time course for kinase activation (maximum in minutes to 1 h) versus proliferation (maximum after days) reflects the difference in the way the assays are performed. Kinase assays on monolayer cultures are designed to detect an EGF response in a 'synchronized' population i.e. re-exposed to EGF after a period in its absence. Under these conditions, a response to EGF is measurable and illustrative of the effect of EGF. During long term continuous exposure to EGF in proliferation assays, we assume a similar activation is occurring (also accompanied by a prolonged elevated basal level) in dividing cells over time as cells enter the proliferating cell pool (represented by cells at the periphery of colonies or monolayers).

Immunoblot analysis of cell lysates was performed using antisera against phosphorylated and total ERK1 and ERK2 to examine the relative activation of ERK1 and ERK2. Shown in Figure 3 is a comparison of normal (A) and tumor cells (B) cultured in basal medium or stimulated by EGF. For both cell types, ERK1 and ERK2 were activated with no apparent change in total ERKs. Examination of the immunoblotting data suggests that in tumor cells, EGF activates ERK1 (p44) to a greater extent than ERK2 (p42). Densitometry of the ERK bands and comparison of the ratio (ERK1:ERK2) of the fold increases caused by EGF at 4 min showed that this ratio was

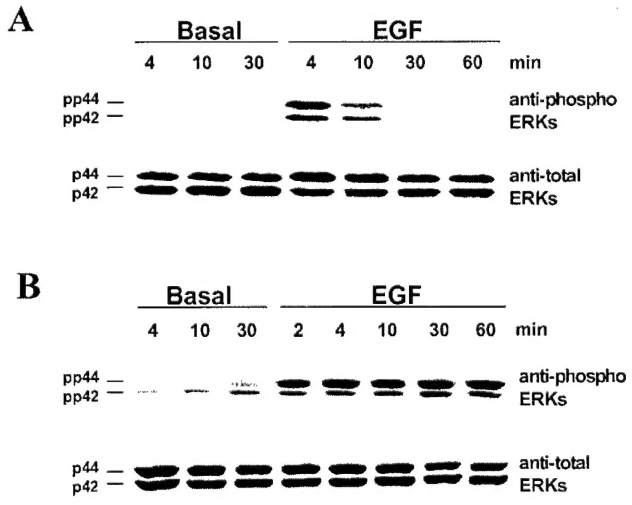

Fig. 3. Western blot analysis of ERK 1 and ERK 2 activity in (**A**) normal and (**B**) tumor mammary epithelial cells. Cell cultures were treated with EGF (10 ng/ml) for 2, 4, 10, 30 and 60 min prior to termination. Basal (B) control cultures were done in parallel. Cell lysates were electrophoresed, transferred to nitrocellulose membranes and blotted with phospho-specific ERK antisera (activated ERKs, top panels) followed by stripping and reprobing with ERK 1 and ERK 2 antisera (total ERKs, lower panels). Results are representative of three experiments for normal and tumor cells.

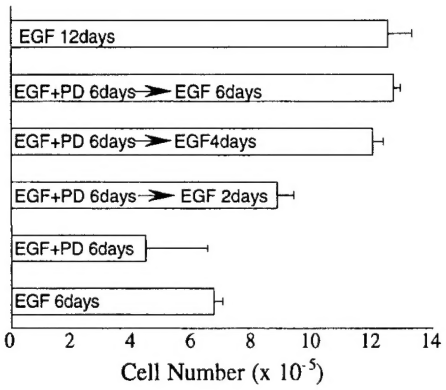

Fig. 6. Reversibility of PD 098059 growth inhibition in tumor cells. PD was added to EGF-stimulated cultures for 6 days (EGF + PD 6 days). At this time, parallel cultures in PD were switched to EGF-only medium and cultured for an additional 2, 4 or 6 days before termination. Control cultures containing only EGF were terminated at 6 days (EGF 6 day) and 12 days (EGF 12 days). Similar results were obtained in a second experiment.

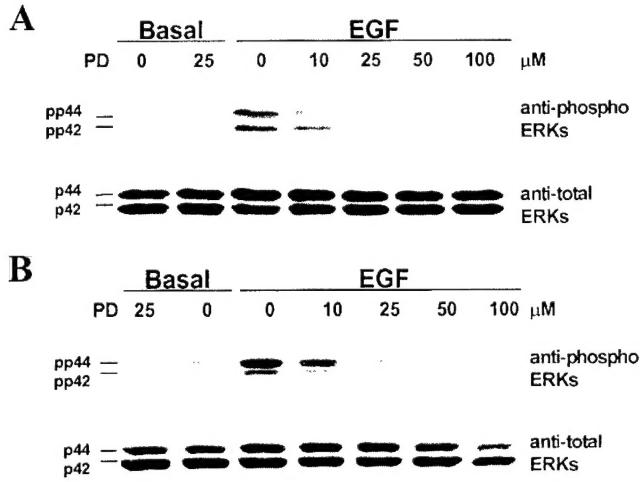

Fig. 7. Effect of PD 098059 on MAP kinase activation in (**A**) normal and (**B**) tumor mammary epithelial cells. Cells were preincubated in inhibitor for 1 h before EGF was added. After 4 min in EGF, cultures were terminated and western blotting for phosphorylated and total ERKs was performed as described in Figure 3. Representative of three experiments.

cells were cultured in the presence and absence of this compound and ERK activation was assessed by electrophoresis and immunoblotting of cell lysates with antisera to phosphorylated ERK1 and ERK2. Figure 7 shows that ERK phosphorylation was inhibited in normal and tumor cells in a concentration dependent manner with complete inhibition of EGF-stimulated ERK phosphorylation at 50–100 μM.

EGF receptor phosphorylation in normal and tumor cells

As shown in Figure 1, maximum EGF-stimulated ERK activation was prolonged in the tumor and relatively transient in normal cells. EGF binding to its receptor results in receptor dimerization and transphosphorylation of tyrosines by the receptor kinases (4). We monitored tyrosine phosphorylation of the receptor to compare the time course of receptor activation to ERK activation. Figure 8 shows that the time course of EGF-induced receptor autophosphorylation differed between normal and tumor cells. Receptor phosphorylation was sustained in tumor but not normal cells where it declined significantly by 30 min. Thus, we find a correlation between ligand-induced EGF receptor activation and ERK activation, sug-

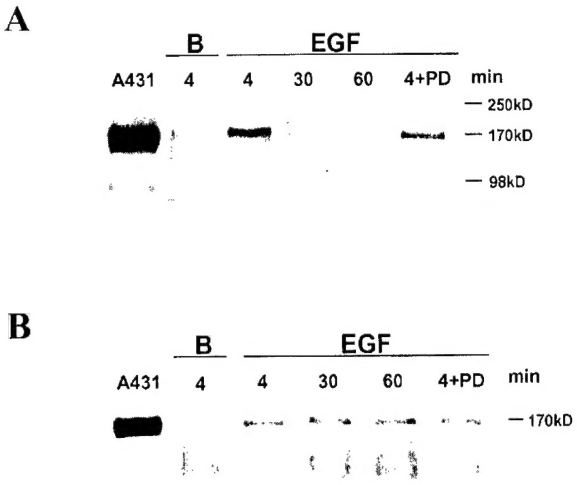

Fig. 8. Phosphorylation of the EGF receptor in response to EGF and PD 098059. (**A**) Normal and (**B**) tumor mammary epithelial cell cultures were stimulated by EGF for 4–60 min. PD 098059 was also included in parallel for cells exposed to EGF for 4 min (peak response). Cell lysates were immunoprecipitated with anti-phosphotyrosine antisera then the immunoprecipitates were subjected to western blot analysis with EGF receptor antisera. The 170 kDa band is the phosphorylated EGF receptor. A431 cell lysates were run as a positive control. Representative of three experiments. B, basal untreated culture.

gesting that the extended time course of activation in tumor cells is due to continued stimulation of the ERK pathway initiated at the EGF receptor. Figure 8 also shows that PD 098059 pretreatment of cells did not block EGF receptor autophosphorylation (or EGF-induced c-jun kinase (JNK1 and JNK2) activation (unpublished observation)), in agreement with the reported specificity of this agent. Western immunoblotting of cell lysates with antisera to the EGF receptor showed that there was no apparent change in total receptor level during a 1 h time course in normal and tumor cells (data not shown).

Discussion

These studies were initiated to examine the hypothesis that the regulation of the activity of the MAP kinases, ERK1 and ERK2 could be altered during mammary tumorigenesis. This hypothesis was tested by comparing the effect of EGF, a known activator of this pathway, on proliferation and activation of ERKs in normal and tumor mammary epithelial cells. Previous work examining the effect of growth factor stimulation of confluent and quiescent cultures of fibroblasts has shown that after an early peak in ERK activation, there is a slower elevation in activity over several hours to a level above basal. This later rise or sustained increase in activity is associated with the stimulation of DNA synthesis (22–25). The time course of ERK activation in our primary epithelial cell cultures differs from that observed in fibroblasts by lacking this biphasic quality. In normal mammary epithelium, EGF stimulates peak ERK activity at 4–5 min post-exposure, with a decline to a steady level ~2-fold above basal by 30 min; this is presumably sufficient and necessary to maintain mitogenesis as revealed using the upstream MEK inhibitor, PD 098059. Thus, during proliferation occurring over a period of 7–10 days, the continuous presence of EGF would stimulate ERK activity in the proliferating pool of cells shown to be localized at the periphery of the colonies or tips of growing projections (26).

In the tumor cells, EGF stimulation caused ERK activity to rise to a plateau at 4–5 min before declining slowly but

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